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Susceptibility and Protection of Naïve and Vaccinated Racing Pigeons (*Columbia livia*) Against Exotic Newcastle Disease Virus from the California 2002–2003 Outbreak

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SUMMARY. The susceptibility, immune response, and protection to challenge after vaccination in racing pigeons (*Columbia livia*) was assessed with the 2002–2003 exotic Newcastle disease (END) virus responsible for the most recent major outbreak in Southern California. Immunologically naïve pigeons appeared resistant to disease, regardless of dose, after a natural route of exposure. Twenty percent morbidity was observed in each group of birds receiving between $10^{2.1}$ and $10^{8.1}$ 50% embryo infectious dose (EID_{50}) per bird, with one bird succumbing to challenge in the $10^{8.1}$ EID_{50}/bird group at day 12 postinoculation. Although resistant to disease, birds in all groups continued to shed virus from either oral or cloacal route at the end of the 14-day sampling period, and seroconversion was only observed in birds receiving $\geq 10^{6.1}$ EID_{50}. Single or double vaccination of juvenile and adult birds with pigeon paramyxovirus virus type 1 (PPMV-1) vaccine followed by END challenge with $10^{6.1}$ EID_{50}/bird decreased the duration, incidence, and viral load. A positive correlation was observed between the presence of hemagglutination-inhibiting antibody titers at challenge and decreased viral shedding. Overt clinical signs of disease were not observed in any PPMV-1–vaccinated birds after challenge.

RESUMEN. Susceptibilidad y protección de palomas de carrera (*Columbia livia*) vacunadas o no contra el virus de la enfermedad de Newcastle del brote de California 2002–2003.

Utilizando el virus de la enfermedad de Newcastle responsable del mas reciente brote de la enfermedad de Newcastle en el sur de California (2002–2003), se evaluó la susceptibilidad, respuesta inmune y protección al desafío en palomas de carrera (*Columbia livia*). Las palomas sin exposición previa aparentaron ser resistentes a la enfermedad independientemente de la dosis de exposición al virus por las rutas naturales. Se observó una morbilidad del 20% en cada uno de los grupos de aves que recibieron entre $10^{2.1}$ y $10^{8.1}$ dosis infectiva 50% (DI_{50}) para embrión de pollo/por ave, una de las aves sucumbió al desafío 12 días posteriores a la inoculación en el grupo de $10^{8.1}$ DI_{50} para embrión de pollo. Aunque resistentes a la enfermedad, las aves en todos los grupos continuaron la diseminación del virus por vía oral o cloacal hasta el final de los 14 días del periodo de muestreo y solo se observó seroconversión en las aves que recibieron virus con títulos $\geq 10^{6.1}$ DI_{50} para embrión de pollo. La vacunación simple o doble de aves jóvenes y adultas con el paramixovirus tipo 1 de paloma disminuyó la duración, incidencia y carga viral de un desafío con virus de la enfermedad de Newcastle ($10^{6.1}$ DI_{50} para embrión de pollo). Se observó una correlación positiva entre la presencia de títulos de anticuerpos inhibidores de la hemoaglutinación al momento del desafío y la disminución de la diseminación del virus. Posterior al desafío, no se observaron signos clínicos evidentes de la enfermedad en ninguna de las aves vacunadas con el paramixovirus tipo 1 de paloma.

Key words: exotic Newcastle disease, pigeon, vaccine, veterinary virology, immunology, avian paramyxovirus

Abbreviations: C = challenged; ED = eye drop; EID_{50} = 50% embryo infectious dose; END = exotic Newcastle disease; ENDV = END virus; HA = hemagglutination; HI = hemagglutination inhibition; IN = intranasal; i.v. = intravenous; NC = nonchallenged; ND = Newcastle disease; NDV = ND virus; PBS = phosphate-buffered saline; PC = postchallenge; PI = postinfection; PPMV-1 = pigeon paramyxovirus; RRT-PCR = real-time reverse transcription polymerase chain reaction; SC = subcutaneous; SPF = specific pathogen free; VI = virus isolation; vNDV = virulent NDV; woa = weeks of age

Newcastle disease virus (NDV) is classified as a member of the *Avulavirus* genus, within the *Paramyxoviridae* family. NDV isolates have been classified as lentogenic (low), mesogenic (moderate), or velogenic (highly virulent) depending on the severity of disease produced by the isolate in chickens (1). The occurrence of highly virulent NDV (vNDV) infections are recognized as a notifiable disease reportable to the World Organization of Animal Health (Office of International Epizooties) (17). In the United States, outbreaks of vNDV in poultry are termed exotic Newcastle disease (END).

During October 2002, END was isolated from backyard game chickens in Southern California (Los Angeles county) (14), which preceded diagnosis of END in commercial poultry in December 2002 (15,16,18). More than 3 million birds, including approximately 150,000 backyard flocks, were depopulated at an estimated cost of $250 million during the END outbreak. Phylogenetic analysis of this virus has determined it is most related to a vNDV outbreak in Mexico in 2000 (18).

The ability of nonpoultry avian species to introduce and disseminate NDV has been established previously (8) and is believed to have played a major role in the 1971–73 California (Fontana) END outbreak (1,20). In addition, wild bird species including ducks and geese infected with END might not display clinical signs of disease, resulting in an unrecognizable ''carrier'' state that is a major concern of the poultry industry (1).

Feral pigeons (*Columbia* spp.) previously have been implicated as carriers of vNDV (4,7,13,19). Diagnostic sampling with the use of a newly developed real-time reverse transcription polymerase chain reaction (RRT-PCR) test during the most recent California END outbreak indicated that 0.21% (12/5740) of all pigeons tested were positive for END (10). However, the majority of END-positive pigeons were either from multiple species locations or from premises where END was concurrently isolated from poultry.

In these studies, a dose titration challenge was performed to determine the susceptibility of racing pigeons to END recovered during the 2002–2003 California outbreak. In addition, protection

Table 1. Clinical signs of disease after CA02 dose titration in pigeons.

Dose (EID_{50}/bird)	*n*	Number of birds displaying clinical signs[A] days after challenge[B]									% Mortality	% Morbidity
		10	11	12	13	14	15	16	17	18		
$10^{2.1}$	5	1	1	1	1	1	1	1	1	—	0	20
$10^{4.1}$	5	—	—	—	—	—	1	1	1	1	0	20
$10^{6.1}$	5	1	1	1	1	1[C]	—	—	—	—	0	20
$10^{8.1}$	5	1	1	1[D]	—	—	—	—	—	—	20	20
Control	5	—	—	—	—	—	—	—	—	—	0	0

[A]Clinical signs included body tremors, circling, incoordinated movements, paralysis, opisthotonos, or a combination of signs.
[B]No clinical signs were observed in any group before day 10.
[C]Bird with nervous tremors was euthanized and necropsied to obtain samples for histopathology. No increase in severity of tremors had been noted.
[D]Bird euthanized—initially observed nervous body tremors had progressed to paralysis.

extended by vaccination, either singly or doubly, with a commercially available pigeon paramyxovirus (PPMV-1) vaccine was determined after END challenge. The ability of these birds to disseminate END was determined by measuring duration and amount of virus shedding from oral and cloacal swabs by virus isolation (VI) in embryos. Finally, oral swabs from END-infected unvaccinated pigeons were tested with a previously described NDV RRT-PCR test and compared with VI (21).

MATERIALS AND METHODS

Pigeons. Pigeons used in these studies were provided by the American Racing Pigeon Union (www.pigeon.org). Birds used within an experiment were obtained from the same loft; however, birds in experiments I and II were not from the same loft. Birds were received at Southeast Poultry Research Laboratory (SEPRL) and immediately transported to a biosafety level 3 agriculture unit for a minimum 3-day acclimation before challenge (5). Sera were taken from five birds before group randomization in experiment I for NDV antibody testing before challenge. All birds were maintained in either Horsfall isolation units or Mark IV CIS isolation cabinets with a wooden perch, pigeon feed (Global Pigeon Supply, Savannah, GA), grit (Global Pigeon Supply), and water *ad libitum.* In addition, all birds received care under strict guidelines of the SEPRL Institutional Animal Care and Use Committee established specifically for *Columbia livia* species. Euthanasia was performed by the administration of sodium pentobarbital given intravenously at a dose of 100 mg/kg weight.

Viruses. A velogenic strain of END, California 2002 (CA02; game chicken/US[CA]/S0212676/02), was used for all experiments. This isolate was responsible for a recent epizootic outbreak in the southwestern United States recovered from a game bird in California during October 2002 (14). PPMV-1 strain Pigeon 84 (pigeon/U.S. [NY]/84) (11) was used as homologous antigen for HI testing in experiment II. NDV was propagated and titrated in 9–11-day-old SPF chicken embryos via the chorioallantoic sac route.

Experimental design. The initial experimentation was designed to assess susceptibility of pigeons against challenge from CA02 and determine level and duration of viral shedding. Subsequently, single or double vaccination of juvenile or adult pigeons with a commercially available PPMV-1 vaccine was investigated for protection from CA02 challenge. Humoral antibody response and reduction and duration of viral shedding were determined.

Experiment I. Twenty-five pigeons (10–20 wk of age [woa]) were arbitrarily divided into five groups of five birds. Birds in group 1 (Control) received 100 μl of phosphate-buffered saline (PBS, pH 7.4) via eye drop (ED; 50 μl) and intranasal (IN; 50 μl) routes. Birds in groups 2, 3, 4, and 5 received $10^{2.1}$, $10^{4.1}$, $10^{6.1}$, or $10^{8.1}$ CA02, respectively, via ED/IN as described above. After inoculation, birds were monitored daily for overt clinical signs of disease (edema, muscular tremors, torticollis, paralysis of wings and legs) and mortality for 18 days. Pigeons displaying severe clinical signs of disease were euthanized by overdose of sodium pentobarbital as described above. Serum samples were taken by wing bleed at 0 and 14 days postinoculation (PI). Oropharyngeal swabs were collected by calcium alginate swabs (Fisher Scientific, Atlanta, GA), placed into 2-ml brain–heart infusion broth with antibiotics (1000 units/ml penicillin G, 200 μg/ml gentamicin sulfate, and 4 μg/ml amphotericin B; Sigma Chemical Company, St. Louis, MO) from each bird on days 0, 2, 4, 8, and 14 PI and kept at –70 C for VI. END-positive swabs were diluted and titrated as described below to determine viral load.

Experiment II. Thirty pigeons were divided into six groups of five birds. Birds in groups 1 (Control-nonchallenged [NC]) and 2 (Control-challenged [C]) were sham-vaccinated and received 100 μl of PBS injected subcutaneously (SC) under the skin of the neck and challenged at 25 woa. Birds in group 3 (adult-single) received single vaccination at 16 woa and challenge at 25 woa. Birds in group 4 (juvenile-single) received single SC vaccination at 4 woa and challenge at 12 woa. Birds in group 5 (adult-double) received vaccination at 16 and 18 woa age, followed by challenge at 25 woa. Birds in group 6 (juvenile-double) received vaccination at 4 and 6 woa, followed by challenge at 12 woa. All PPMV-1 vaccines were applied via SC route by the pigeon grower before arrival at SEPRL. All END challenge was performed via the ED/IN route with $10^{6.1}$ EID_{50}/bird CA02 in 0.1 ml. Birds in group 1 were kept as unchallenged controls. After challenge, birds were monitored daily for clinical signs of disease and mortality for 14 days, with serum and swabs processed as described above.

Hemagglutination (HA) and hemagglutination inhibition (HI) assays. The HA and HI tests were performed by standard microtiter plate methods. The HI tests were performed as previously described, with four HA units per well (12). In experiment II, PPMV-1 was used for comparison as homologous antigen with LaSota antigen in the HI test as described above.

Virus isolation and titration. VI procedures in embryonated chicken eggs followed standard protocols (2). Virus titers were calculated after inoculation of 10-fold dilutions into 9- or 10-day-old embryonated chicken egg as previously described (12).

RRT-PCR. Oropharyngeal swab samples taken for VI in experiment I were analyzed for detection by RRT-PCR with a modified procedure from previously published protocols with M gene primers/probes specific for END (21). A notable substitution of smaller calcium alginate swabs (Fisher Scientific) were used in this study compared with larger cotton swabs used in the referenced protocol because of the smaller size of birds used in these studies.

RESULTS

Experiment I: Susceptibility of pigeons to CA02. Susceptibility to END challenge was determined by presence of clinical signs during the 18-day PI observation period. No clinical signs of Newcastle disease were observed in any pigeons before challenge. In addition, no clinical signs were observed in any group receiving END inoculation before day 10 PI (data not shown). Birds

Table 2. Mean hemagglutinin inhibition antibody titers[A] after dose titration of CA02 in racing pigeons in Experiment I.

Dose (EID_{50}/bird)	*n*	Day 0[B]	Day 14
NC	5	<2	<2
$10^{2.1}$	5	<2	<2
$10^{4.1}$	5	<2	2
$10^{6.1}$	5	<2	4.5
$10^{8.1}$	5	<2	5.7[C]

[A]Geometric mean titer (log_2). Titers >4 considered positive.
[B]Five pigeons were arbitrarily bled as a representative of all birds used in the experiment.
[C]One bird died in this group on day 12 ($n = 4$).

in the unchallenged (Control) group displayed no clinical signs during the course of the experiment (Table 1). One pigeon receiving a $10^{2.1}$ dose displayed transient mild tremors on days 10–17 PI. One pigeon in the $10^{4.1}$ dose group displayed body tremors with uncontrolled body movements and torticollis, which lasted throughout the testing period. In the $10^{6.1}$ group, one bird displayed general body tremors from day 10 to day 14 PI This bird was necropsied during the course of study for histopathology examination; however, no increase in severity of tremors was noted before euthanasia. One bird in the $10^{8.1}$ group displayed general body tremors and incoordination on day 10 PI, which progressed to paralysis by day 11 PI. This bird was humanely euthanized on day 12 PI.

All sera tested before inoculation were negative to NDV by HI testing (Table 2). Antibodies to NDV were not detected in either the nonchallenged group or the birds receiving $10^{2.1}$ END. Birds receiving $10^{4.1}$ displayed a modest increase in HI titer at day 14 PI. HI antibody titers (log_2) were highest in birds receiving a dose of either $10^{6.1}$ (4.5) or $10^{8.1}$ (5.7).

VI results from swabs taken on days 0, 2, 4, 8, and 14 PI in Experiment I are presented in Table 3. As expected, uninoculated birds were negative for END during the course of study. In general, a positive correlation was observed between the dose of virus inoculum and VI during the course of study. Birds receiving the lower doses of CA02 ($10^{2.1}$ and $10^{4.1}$) had decreased frequency of shedding virus compared with birds receiving higher doses ($10^{6.1}$ and $10^{8.1}$). After virus challenge, the total number of positive birds shedding virus via oral or cloacal routes was 13 of 80 (16%) for the birds receiving the lower doses. In contrast, 38 of 78 (49%) samples were positive from the birds inoculated with the higher doses. Only 3 of 40 (8%) oral swabs were VI-positive from birds receiving the lower dose compared with 17 of 39 (44%) oral swabs from birds receiving the higher doses. Similar observations were observed from cloacal swabs, of which 10 of 40 (25%) or 22 of 39 (56%) were positive in the groups of birds receiving lower or higher doses of CA02, respectively. It is important to note most of the VI-positive swabs were obtained from birds not exhibiting clinical signs of disease and that at least one bird in each group continued to shed virus at the conclusion of the sampling period.

Table 3. Virus isolation in naïve pigeons after dose titration of CA02 from oral and cloacal swabs in Experiment I.

Dose (EID_{50}/bird)	Postchallenge sample (no. positive/total)							
	Day 0		Day 2		Day 4		Day 8	
	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
NC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
$10^{2.1}$	0/5	0/5	1/5	2/5	0/5	2/5	0/5	0/5
$10^{4.1}$	0/5	0/5	1/5	1/5	1/5	1/5	0/5	1/5
$10^{6.1}$	0/5	0/5	4/5	4/5	3/5	3/5	1/5	2/5
$10^{8.1}$	0/5	0/5	3/5	2/5	4/5	3/5	1/5	2/5

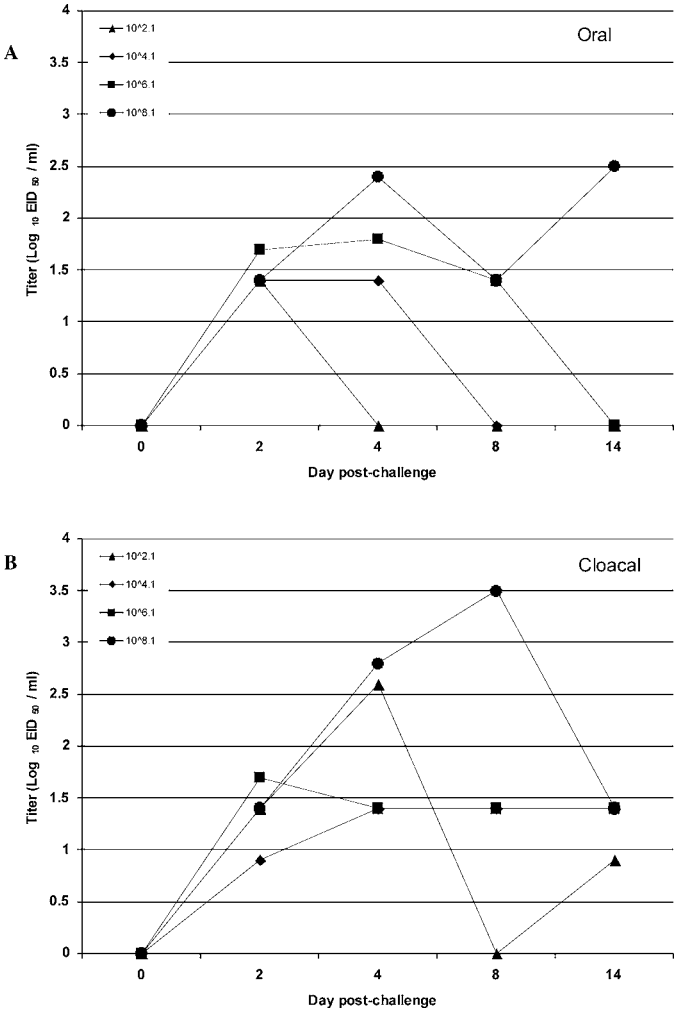

Fig. 1. Comparison of CA02 mean virus titers recovered from oral and cloacal swabs after inoculation with CA02 virus. Pigeons were inoculated with CA02 at $10^{2.1}$–$10^{8.1}$ EID_{50}/bird. Oral (A) and cloacal (B) swabs were sampled on the days indicated.

Low titers of CA02 were isolated from END-positive pigeons from both oral and cloacal swabs (Fig. 1A,B). Viral titers from oral swabs increased early after infection with the highest titers, $\sim 10^{2.5}$ EID_{50}/ml, recovered at day 4 and day 14 PI from birds receiving $10^{8.1}$ EID_{50}/ml. In all groups except $10^{8.1}$, viral titers from oral swabs were $<10^2$ EID_{50}/ml. A positive correlation was observed between the dose of END received and the titer and duration of shedding in oral swabs. Birds receiving the higher doses shed higher titers longer than birds receiving lower doses. For groups receiving low doses of END, viral titers ranged from $10^{0.9}$ to $10^{1.5}$ EID_{50} on all days tested. Cloacal swab titers increased in all groups between days 2 and 4 PI, with titers ranging from $10^{0.9}$ to 10^2 EID_{50} on all days tested.

Comparison of VI in embryos to virus detection with RRT-PCR. Of the 124 oral swab samples tested by VI and RRT-PCR, 93 were negative by both assays and two were positive by both assays (Table 4). Overall, the assay agreed on 95 samples (76.6%) and disagreed on 29 samples (23.4%). VI detected 30 positive samples, two of which were positive by RRT-PCR. Compared with VI, the relative sensitivity of the RRT-PCR test with pigeon samples was 6.7% and the relative specificity was 99%.

Table 4. Comparison of virus isolation in embryos with RRT-PCR for detection of CA02 from oral swabs from pigeons in Experiment I.

	Virus isolation		Total RRT-PCR samples
	+	−	
RRT-PCR			
+	2	1	3
−	28	93	121
Total VI samples	30	94	124

Experiment II: Protective immunity induced in adult and juvenile pigeons receiving single or double PPMV-1 vaccinations against CA02 challenge. No clinical signs of disease were observed in any pigeons before challenge. All birds in the Control-NC group remained normal during the course of the experiment (data not shown). Two of the sham-vaccinated END-challenged birds displayed mild tremors on day 8 postchallenge (PC), which quickly subsided by day 11 PC. No clinical signs of disease were observed in any vaccinated birds, regardless of age at vaccination or number of vaccinations.

Prechallenge sera were obtained from all birds and tested for HI activity with the use of homologous PPMV-1 antigen (Pigeon 84) and compared with standard LaSota antigen. In all cases, higher HI titers were observed when vaccine-homologous antigen was used in the HI test. Sera from nonvaccinated birds tested negative for antibodies to NDV (Table 5) and remained negative after mock-challenge (NC). Nonvaccinated CA02-challenged birds (Challenged) did not exhibit positive HI antibody titers to NDV on day 0 but did exhibit seroconversion on day 14 PC with titers ($\log_2$) of 4.6 and 7.3 with either LaSota or Pigeon 84 antigen, respectively. Juvenile pigeons receiving a single or double dose of PPMV-1 vaccine before challenge exhibited positive HI titers with the use of homologous antigen of 4.6 and 6.6, respectively, at day 0. Before challenge, adult pigeons receiving either single or double vaccination displayed titers of 3.8 and 5.6, respectively. After challenge, titers from all PPMV-1–vaccinated groups increased to >8 by day 14 PC.

VI results from swabs taken on days 0, 2, 4, 6, and 14 PC in Experiment II are presented in Table 6. No virus was isolated before challenge or in the unchallenged group (NC) during the course of the study. All CA02-challenged birds in each group were 100% positive on day 2 PC from oral swabs. Twenty percent of birds (1/5) each from the Challenged and Adult-single groups were virus-positive with cloacal swabs at day 2 PC On day 4 PC, 100% (5/5) of the Control-challenged birds were positive by oral swabs, whereas only 40% (2/5) were positive in each of the vaccinated groups. No cloacal swabs were positive in the vaccinated birds after day 2 PC, regardless of age. Control-challenged birds continued to shed virus by oral swabs on day 8 PC and cloacal swabs on day 4 PC. No virus-positive swabs were observed in either oral or cloacal samples from any group on day 14 PC.

CA02 titers from oral swabs ranged between $10^{2.5}$ and $10^{3.1}$ EID_{50}/ml from all groups challenged on day 2 PC (Fig. 2A). Viral titers decreased to between 10^{1} and $10^{1.4}$ EID_{50}/ml in all groups by day 4 PC. For cloacal swabs, low titers ($<10^{1}$ EID_{50}/ml) were recovered from all groups on day 2 PC. Increased cloacal titers were observed in the control (Challenged) group only on day 4 PC.

Table 5. Comparison of mean hemagglutination inhibition antibody titers with heterologous LaSota antigen and homologous PPMV-1 antigen after vaccination with a single or double dose of inactivated PPMV-1 vaccine in adult and juvenile pigeons.

	Titer (EID_{50}/bird)[A]			
	LaSota antigen		PPMV-1 antigen	
Group	Day 0	Day 14	Day 0	Day 14
NC	<2	<2	<2	<2
Challenged	<2	4.6	<2	7.3
Juvenile-single	1	6.4	4.2	>8
Juvenile-double	2	4.6	6.6	>8
Adult-single	1.6	7.8	3.8	>8
Adult-double	1.6	6.9	5.6	>8

[A]Geometric mean titer ($\log_2$). Titers >4 considered positive.

DISCUSSION

In this study, racing pigeons were exposed to END virus (ENDV) isolated during the recent California outbreak to determine susceptibility to disease and potential to shed END after a natural route of exposure. Although no data exist on dose size after natural exposure in the field, it is believed the higher doses used in these studies would likely exceed the dose birds would encounter in the wild. Immunologically naïve racing pigeons appeared resistant to disease on the basis of infrequently observed clinical signs, in agreement with previously published results obtained from pigeons infected by a similar route and dose of exposure (4,7). Using ENDV isolated during the 1971 California (Fontana) outbreak, Erickson *et al.* (7) established an age-dependent susceptibility of unvaccinated juvenile and adult racing pigeons. After intraocular route of exposure with $10^{6.1}$ EID_{50} Fontana, 10% and 33%, respectively, of adult and juvenile racing pigeons succumbed to challenge. The majority of those birds were determined to shed virus at the earliest time point taken (3 days PI). A positive relationship was observed between the youth of the birds and length of virus shedding. Juvenile birds were shown to shed Fontana virus up to 21 days PI, whereas adult birds only shed virus up to 7 days PI. Although direct comparisons of age-related effect on pigeons between the Fontana and CA02 ENDV cannot be made because it is unclear what the age of distinction between juvenile and adult was used in that study, the majority of pigeons used in susceptibility studies presented here were approximately 12 woa or less, which might explain the differences in shedding patterns observed between Control-challenged birds in experiments I and II. In experiment I, birds receiving $10^{6.1}$ END continued to shed virus from both oral and cloacal swabs on the last sampling day, whereas Control-challenged birds from experiment II (25 woa) did not display virus-positive swabs beyond day 8 PI. These results support the age-dependent susceptibility of racing pigeons to END.

Alexander *et al.* (4) determined that route of infection appeared to be critical for reproduction of clinical disease because pigeons inoculated intravenously (i.v.) were susceptible to increased mortality. However, those studies were performed with a virulent PPMV-1 isolate in contrast to the END chicken isolate used in these studies. Whether i.v. inoculation of CA02 increases mortality remains to be determined. Unvaccinated pigeons from that study were observed to shed PPMV-1 up to 16 days PI, and initial VI from cloacal swabs was not observed until day 6 PI. The studies presented here detected virus from both oral and cloacal routes immediately after inoculation, and virus-positive swabs were detected at the termination of the experiment.

Humoral immune response to END inoculation and number of birds shedding virus appeared to correlate with dose. Birds receiving lower doses of virus ($\leq 10^{4.1}$ EID_{50}) did not exhibit positive HI titers 2 wk postinoculation, whereas both groups receiving higher doses

Table 6. Virus isolation after vaccination and challenge of juvenile and adult pigeons with CA02 at $10^{6.1}$ EID_{50} from oral and cloacal swabs.

	Postchallenge sample (no. positive/total)									
	Day 0		Day 2		Day 4		Day 8		Day 14	
Group	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal	Oral	Cloacal
NC	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Challenged	0/5	0/5	5/5	1/5	5/5	2/5	1/5	0/5	0/5	0/5
Juvenile-single	0/5	0/5	5/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5
Juvenile-double	0/5	0/5	5/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5
Adult-single	0/5	0/5	5/5	1/5	2/5	0/5	0/5	0/5	0/5	0/5
Adult-double	0/5	0/5	5/5	0/5	2/5	0/5	0/5	0/5	0/5	0/5

($\geq 10^6$ EID_{50}) displayed positive titers ($\geq 2^4$) at the end of the inoculation period. Birds receiving the lower doses also had lower incidence of END-positive swabs. These results suggest that although the birds receiving the lower doses became infected, the antigenic mass resulting from infection did not reach the threshold necessary for seroconversion as assayed in this study.

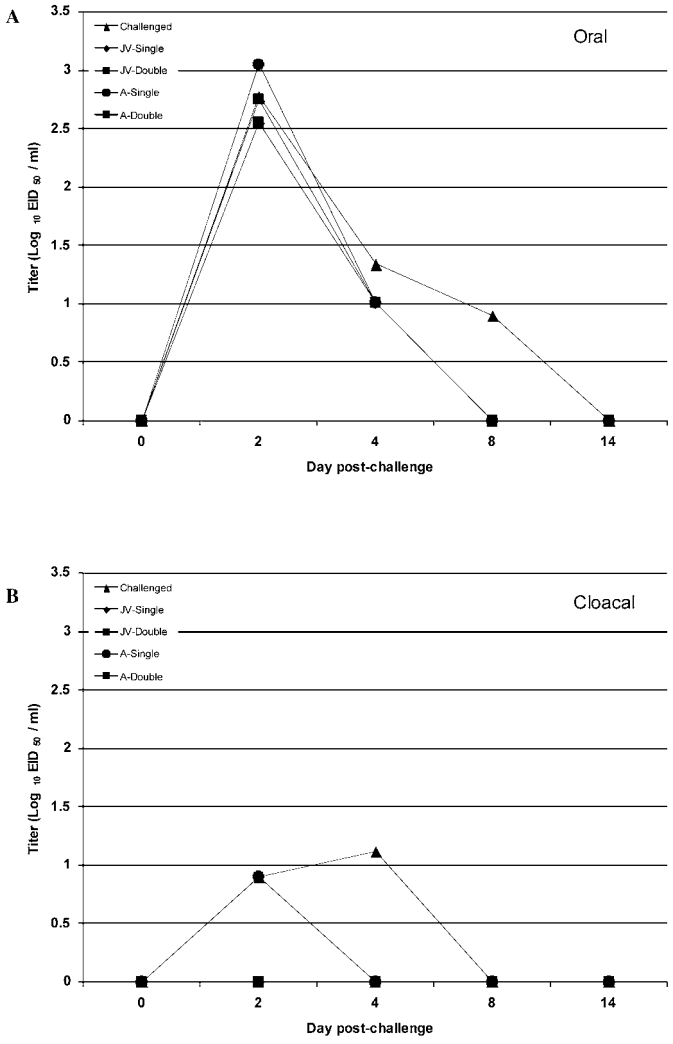

Fig. 2. Reduction of virus shed after vaccination with an inactivated PPMV-1 vaccine in Experiment II. Juvenile-single pigeons received a single SC vaccination at 4 woa and challenge at 12 woa, and juvenile-double pigeons received vaccination at 4 and 6 woa, followed by challenge at 12 woa with CA02 at $10^{6.1}$ EID_{50}/bird. Adult-single pigeons received single vaccination at 16 woa and challenge at 25 woa, and adult-double pigeons received vaccination at 16 and 18 woa age followed by challenge at 25 woa as above. Oral (A) and cloacal (B) swabs were sampled on the days indicated.

In these studies, the use of a molecular technique to detect END in nonpoultry species was not as sensitive as VI. The RRT-PCR procedure used in this study was adapted for pigeons from a test that was validated in chickens. The poor correlation between VI and RRT-PCR emphasizes the need for comparison testing and validation of a procedure for the intended function and that modifications to a validated procedure can result in unacceptable performance of the test. Wise *et al.* (21) indicated that 10^1 EID_{50} could be detected with the M gene primer/probe set. The majority of titers in this study were determined to be $\geq 10^1$ EID_{50}, which was at the lowest level of detection for the M gene test. However, the composition of the swabs (calcium alginate) and smaller size of the swabs used in these studies might have contributed to the lack of virus detection by the real-time procedure. Cloud *et al.* (6) reported that PCR reactions were inhibited when calcium alginate swabs were used for detection of *Bordetella pertussis*. In addition, the lower limits of detection for the RRT-PCR test were taken from 10-fold dilutions of ENDV in buffer. The samples tested here were from birds and most likely contained competing host and bacterial RNA species that might have affected RNA isolation and thus further decreased the sensitivity of the test in these studies. Further work is needed to optimize and validate the RRT-PCR procedure in pigeons before it can be used as an alternative to virus isolation.

The ability of a commercially available PPMV-1 vaccine to protect against disease and limit viral shedding was also determined. None of the vaccine treatment groups were shown to resist infection after END challenge, although vaccination decreased both the duration and amount of virus shed compared with unvaccinated birds. These results are not surprising because we had previously shown that NDV-vaccinated chickens are not resistant to infection after challenge with the CA02 virus but display reduced viral titers and duration of shedding (9).

Humoral immune response after PPMV-1 vaccination resulted in seroconversion of the majority of adult and juvenile pigeons when homologous antigen was used for HI testing but not NDV (LaSota) antigen. Antibody response to vaccination was dose dependent, regardless of age. After END challenge, all birds displayed increased HI antibody titers, although higher titers were observed with homologous vaccine antigen compared with NDV. These results underscore the antigenic differences and corresponding titer differences previously observed between PPMV-1 and NDV (3).

The continued outbreaks of velogenic NDV emphasize the importance for continued research on vaccine efficacy against newly isolated strains in all species of birds. It is obvious that vaccination of pigeons during outbreak situations would greatly reduce the incidence, duration, and amount of virus shed compared with immunologically naïve birds. However, because both vaccinated and nonvaccinated birds rarely displayed overt clinical signs of disease

after END infection, only diagnostic testing of individual birds should be used to confirm viral status in the event that pigeon races be held in geographic regions of an END outbreak.

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